LASER RAMAN SCATTERING AS ❖956
A PROBE OF PROTEIN STRUCTURE

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PERSPECTIVES AND SUMMARY

The Raman spectrum of a protein shows a series of peaks, characteristic of the molecular groupings present, whose frequencies are sensitive to local structural influences. While Raman intensities are intrinsically low, good quality protein spectra can be obtained with modern Raman spectrometers equipped with laser light sources. The sample can be studied in any physical state; protein spectra are obtainable in situ in biological materials, although background scattering and fluorescence from other components may interfere. The increasing tunability of laser sources makes possible the systematic exploitation of resonance Raman spectroscopy, in which the laser is tuned to an absorption band of the sample. Large enhancements are observed for certain Raman bands of the chromophore.

The instrumentation for normal and resonance Raman spectroscopy is the same, although the latter requires special precautions associated with side effects of light absorption. The information content is quite different, however. The normal Raman spectrum contains all of the active vibrational modes of the molecule (those associated with a change in molecular polarizability). These modes coalesce into recognizable bands for repeating structural units, i.e. peptide links and side chains of a given type. The normal Raman spectrum can therefore be used to study secondary and tertiary structure in proteins. In resonance Raman spectroscopy, enhancement of chromophore vibrations insures that they dominate the spectrum. Often they are the only features observable. Information about nonchromophoric parts of the protein is lost. What is gained, however, is a large increase in sensitivity and selectivity for vibrations of the chromophoric group. They can be monitored at high dilution or in complex mixtures. If, as is often the case, the chromophore is itself a site of biochemical activity, then the resonance Raman spectrum can be used to probe associated structural changes.

While normal and resonance Raman spectra show dramatic differences, there is actually a continuum of spectra between the two limiting cases. Pre-resonance enhancement is observed well outside of the absorption band proper. For example, the high relative intensities of ring vibrations of aromatic residues in Raman spectra excited in the visible region, is associated with proximity to their ultraviolet absorption bands. In principle, one can adjust the selectivity of the Raman spectrum by tuning the laser to different parts of the electronic spectrum. This potential is currently limited by the tuning range of practical laser sources, which is expanding steadily.

INTRODUCTION

Dr. Bull: What future do you hold out for the study of proteins by this technique?

Dr. Edsall: I believe there is hope for the study of proteins, but it is extremely difficult. . . .

Cold Spring Harbor, 1938 (1)

Twenty years elapsed before Professor Edsall's guarded optimism was rewarded with a "quite faint" 18-line photographic Raman spectrum of lysozyme (2); it was to be another ten years before Tobin (3) reported the first laser-excited spectra of lysozyme, pepsin, and α-chymotrypsin. Within two years of Tobin's note the pioneering papers by Lord & Yu (4, 5) appeared. These papers detailed the spectra of lysozyme, ribonuclease, and chymotrypsin, emphasizing structural interpretations, and provided much of the impetus and direction for ensuing Raman studies of proteins. Also in 1970 Rimai and co-workers showed that resonance Raman (RR) spectra of carotene and lycopene can be obtained in situ in carrot and tomato tissue (6) and, in addition, produced the first RR spectrum of rhodopsin in frozen bovine retina (7). At the same time Long & Loehr (8) reported the first RR spectrum of a metalloprotein, rubredoxin. In 1972, RR spectra of hemoglobin and cytochrome c were published (9–11), setting in motion numerous RR studies of heme proteins and metalloporphyrins.

The procedure of Raman studies is to obtain and assign frequencies associated with molecular vibrations of interest. The interpretation of vibrational frequencies in terms of atomic masses, bond distances, and bond angles has firm theoretical and empirical underpinnings from extensive studies of small molecules (12–14). Since many vibrational modes are fairly well localized on specific groups of atoms, extension of the method to large molecules is straightforward, although complex. The potential for providing important information on biomolecular structure is high.

Two forms of spectroscopy, infrared and Raman, are capable of providing the needed data. Infrared spectroscopy involves direct absorption of light at frequencies appropriate for molecular vibrations. Since water is a strong absorber of infrared radiation over much of the spectral range of interest, the use of infrared spectroscopy in biological studies is limited, although several important applications, utilizing "window" regions of the water spectrum, have been reported (15–19). Raman spectroscopy involves a scan of the frequency of scattered light using a high-energy monochromatic source. Energy can be transferred to a vibrational transition of

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the sample, with a corresponding frequency shift of the scattered photons. This is a low-probability event and a large incident photon flux is required to produce detectable Raman signals. In the pre-laser days, heroic measures were needed to provide such a flux, usually via gas-discharge lamps surrounding the sample. Large volumes of optically transparent material were required, severely constraining the applicability of the technique. Introduction of the laser, with its highly concentrated beam of light, dramatically relaxed these constraints. It is now possible to obtain a Raman spectrum of microliters of sample in any physical state (20, 21). Water is a weak Raman scatterer and produces minimal interference in aqueous medium studies.

The following sections summarize selected results of recent protein Raman studies. The field is burgeoning, and it is impossible in the space available to provide a comprehensive review. We hope instead to give some flavor for the kinds of information available and the potential for future work. More detailed reviews of both normal (22) and resonance Raman (23) work in this area have recently been published. As mentioned in the summary, quite different information emerges from these two approaches, yet the potential exists for combining them by appropriate laser tuning. The recent investigation by Shimanouchi and co-workers (24) of pre-resonance enhancement of peptide modes, using a series of visible and ultraviolet laser lines, is an apt example.

RAMAN SPECTRA AND PROTEIN CONFORMATION

Although the vibrational modes of a protein are exceedingly numerous, a normal protein Raman spectrum shows consistent and recognizable peaks, as illustrated for carbonic anhydrase in Figure 1. The peak intensities depend on the number of repeating vibrational units in a given chemical environment and on the change in molecular polarizability associated with the vibration. Both the peptide backbone and the amino acid side chains contribute. Assignment of the bands rests on extensive comparisons with amino acid and peptide spectra (4, 5, 22). Some of the bands have been found empirically to be sensitive, with respect to frequency and/or intensity, to conformational changes. These are summarized in Table 1, and a brief discussion is given below.

Peptide Backbone

Of the vibrational modes associated directly with the amide bond, two have sufficient Raman intensity to be generally useful. Amide I, occurring in the region 1630–1690 cm^{-1}, involves peptide $C=O$ stretching primarily, while amide III, at 1225–1275 cm^{-1}, has contributions from both C–N stretching

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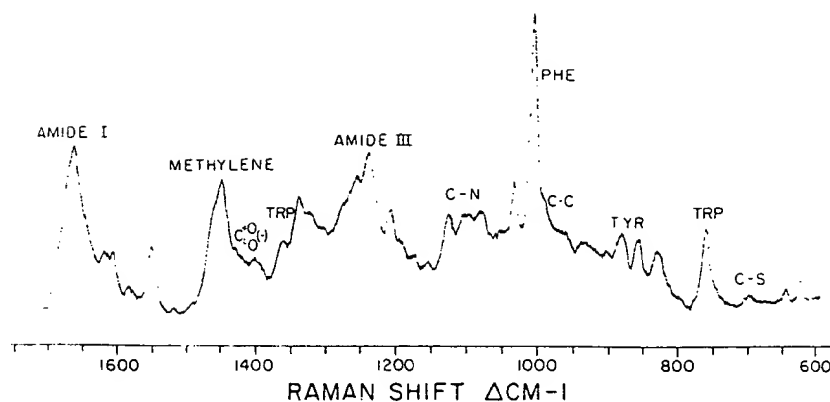etching primarily, while
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Figure 1 Laser Raman spectrum of a 14-mM solution of human carbonic an-
hydrase-B. Several modes useful in structural interpretation are identified (see
Table 1).

and N–H in-plane bending. Model studies with homopolypeptides (22,
24–27) and proteins of well-defined structure (28–33) show that both fre-
quencies are sensitive to peptide conformation, and establish the follow-
ing assignments: anti-parallel β-pleated sheet, $\sim$1670 cm^{-1} (I) and $\sim$1235
cm^{-1} (III); α-helix, $\sim$1655 cm^{-1} (I); disordered structure, $\sim$1665 cm^{-1} (I)
and $\sim$1245 cm^{-1} (III). The amide III frequency for the α-helical conforma-
tion is still controversial. It had been thought to occur at $\sim$1270 cm^{-1}, but
both histidine and tyrosine contribute to this region (31). It is now clear that
α-helix is characterized by the absence of intensity below 1275 cm^{-1} (25,
29) and by the presence of a strong band around 1310 cm^{-1} (26, 30, 33),
although the proximity of this band to overlapping CH$_2$ deformation modes
makes assignment difficult. Amide I falls in one of the few regions of the
Raman spectrum where scattering from water interferes, and unequivocal
assignment must be made in D$_2$O. The characteristic amide intensities for
the different peptide structures have been related to parallel ultraviolet
absorbance changes via a pre-resonance mechanism (35).

There is much interest in using the Raman spectrum for protein confor-
mational analysis, by resolution of the amide I and III bands. Several
attempts at quantitative estimation of secondary structure have been made
(30, 33, 36). Lippert et al (33) report reasonable success ($\pm$10–15% agree-
ment with published values) using a procedure involving comparison of
protein parameters (in both H$_2$O and D$_2$O) with corresponding data for
polylysine in its α-helical, β-sheet, and random forms.

In addition to the amide modes, skeletal modes involving C–C ($\sim$900–
1000 cm^{-1}) and C–N ($\sim$1100 cm^{-1}) stretching are somewhat sensitive to

Table 1   Raman modes useful in the interpretation of protein structure

Origin and frequency (Δcm^{-1})	Assignment	Structural information	References
Backbone			
Skeletal Acoustic			
25–30	Mode of large portion of protein; possibly inter-subunit mode	Overall structure; subunit interactions	39, 40
75	Torsion mode	α-helix (tentative)	40
160	Bending mode	β-structure (tentative)	40
Skeletal Optical			
935–945 (950 D_2O)	C–C stretch (or Cα–C–N stretch)	α-helix	26, 30, 37 29–31
900	C–C stretch	Conformational change markers broaden and lose intensity with denaturation	31, 38,
963	–		51
1002 (H_2O)	Cα–C' or Cα–Cβ stretch	Suggestive of β-pleated sheet	26
1012 (D_2O)	–	–	
1100–1110	C–N stretch	Conformational change marker broadens and loses intensity with denaturation	51
Amide I	Amide C=O stretch	A sharp band suggests homogeneous structure and uniform hydrogen bonding. Strong hydrogen bonding lowers amide I frequencies	25, 26, 30, 67
1655 ± 5	–	α-helix	26
1632 (D_2O)	–	–	–
1670 ± 3	–	Antiparallel β-pleated sheet	25, 26, 33
1661 ± 3 (D_2O)	–	–	26, 67
1665 ± 3	–	Disordered structure (solvated)	5, 67
1658 ± 2 (D_2O)	–	–	26, 30
1685	–	Disordered structure (non-hydrogen bonded)	29, 67
Amide III	N–H in-plane bend, C–N stretch	Strong hydrogen-bonding raises amide III frequencies	27
>1275	–	α-helix, no structure below 1275 cm^{-1}	25, 26, 30, 67
1235 ± 5 (sharp)	–	Antiparallel β-pleated sheet	25, 26, 49, 67
983 ± 3 (D_2O)	–	–	26, 34,
1245 ± 4 broad	–	Disordered structure	5, 26, 29
1235	–	Disordered structure (non-hydrogen-bonded)	29

Left column (fragment)

:n structure

information	References
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:en-bonded)	

Right column

Table 1 *(Continued)*

Amino Acid Sidegroups

Tyrosine

Band	Assignment	Information	References
850/830 doublet	Fermi resonance between ring fundamental and overtone	State of phenolic –OH I_{850}/I_{830} = 9:10 to 10:7, "exposed" H-bond from acidic donor. 10:7 to 3:10 "buried," strong –OH H-bond to negative acceptor	50

Tryptophan

1361	Indole ring	Ring environment. Sharp intense line for buried residue. Intensity diminished on exposure or environmental change. Band contour of doublet near 1360–1380 is sensitive to environment	31, 51, 58
1338	–	–	–

Phenylalanine

1006	Ring breath	Conformation-insensitive Frequency/Intensity reference	4, 5
624	Ring breath	Ratio with tyrosine 664 cm^{-1} to estimate Phe/Tyr	67

Histidine

1409 (D_2O)	N-deuteroimidazole	Possible probe of ionization state, metalloprotein structure, proton transfer	5

-S-S-

510	S–S stretch	*gauche-gauche-gauche.* Broadening and/or shifts may indicate conformational heterogenity among disulfides	45
525	S–S stretch	*gauche-gauche-trans*	–
540	S–S stretch	*trans-gauche-trans*	–

C-S

630–670	C–S stretch	*gauche*	45
700–745	–	*trans*	–

S-H

2560–2580	S–H stretch	Environment, deuteration rate	67

Carboxylic Acids

1415	$C{\overset{O}{\underset{O}{\diagdown\mkern-12mu\diagup}}}$ stretch	State of ionization	4
1730	$C{\overset{O}{\underset{OH}{=}}}$, C=O stretch		4
	$C{\overset{O}{\underset{OR}{=}}}$, C=O stretch	Metal complexation	75

conformation. A band at 935–945 cm^{-1} appears common to helical structures (25, 29–31, 37, 38), while a band at ~1010 cm^{-1} is considered suggestive of β-structure (25).

Proteins also have low-frequency "acoustical" modes, involving coherent vibrations of large portions of the molecule, which are highly sensitive to conformation. They have been observed in chymotrypsin and pepsin (39) and in lysozyme (40). The topic is the subject of an interesting review by Shimanouchi (41).

Side Chains

Several of the more characteristic protein Raman bands are contributed by aromatic side chains or by disulfide links, the high relative intensity presumably arising from pre-resonance enhancement via the ultraviolet absorption bands of these groups.

DISULFIDE   The suggestion by Lord & Yu (4) that the variation in S–S stretching frequency might be useful in determining disulfide dihedral angles in proteins has been a subject of lively debate (42–48). The currently accepted view (44, 46) is that ν_{S-S} is *not* a direct function of S–S dihedral angle in the range 65–85°, but that some dihedral angle dependence may be evident for highly strained disulfides. The observed variations of ν_{S-S} among alkyl disulfides have been explained by Sugeta (45, 46) as a consequence of vibrational coupling between S–S stretching and S–C–C bending modes. Thus ν_{S-S} is sensitive to the conformation about the C–S bond, depending in particular upon conformations in which two hydrogens, one carbon and one hydrogen, or two carbons occupy positions *trans* to the distal sulfur across the C–S bond. These conformations, which may be denoted GGG (gauche-gauche-gauche), GGT (or TGG), and TGT, are associated (45) with ν_{S-S} of 510 cm^{-1}, 525 cm^{-1}, and 540 cm^{-1}, respectively, for aliphatic disulfides. Modifications of the Sugeta correlations have recently been proposed (48) that take account of substituent effects at the β-carbon and deviations from the strict *trans* and *gauche* conformations assumed by Sugeta.

In analogy to ν_{S-S}, C–S stretching frequencies depend upon the conformation about the C–C bond adjacent to C–S (45): a *gauche* rotamer yielding ν_{C-S} ~630–670 cm^{-1} and a *trans* rotamer, ν_{C-S} ~700–745 cm^{-1}. Thus while disulfide and C–S modes are useful as indicators of protein –S–S– conformation, their utility is more limited and their interpretation considerably more complicated than heretofore realized.

AROMATICS   Yu, Jo & O'Shea (49) proposed that the relative intensities of the tyrosine ring modes at 645 cm^{-1}, 828 cm^{-1}, and 853 cm^{-1} could

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indicate whether the residue was "buried" or "exposed." In a joint study from the laboratories of Lord and Shimanouchi, Siamwiza et al (50) explored the proposal made by Yu et al and concluded the following: (*a*) the doublet near 830 cm⁻¹/850 cm⁻¹ results from Fermi resonance between a fundamental and the first overtone of two ring modes; (*b*) the intensity ratio I_{850}/I_{830} is not sensitive to solvent polarity or backbone conformation, but rather is related primarily to the state of the phenolic OH; (*c*) in particular, a strong H-bond by –OH to a negative acceptor results in a low I_{850}/I_{830} ratio, whereas H-bonding between the phenolic oxygen and an acid donor yields a higher ratio; and (*d*) the ratio I_{850}/I_{830} is in the range 9 : 10 to 10 : 7 for exposed tyrosines, whereas for "buried" tyrosines it can be anywhere between 19 : 7 to 3 : 10.

Conformational aspects of certain modes from phenylalanine, tryptophan, and histidine are mentioned in Table 1.

Applications

Protein Raman spectra have been used to monitor structural aspects of denaturation in lysozyme (31, 51–53), β-lactoglobulin (54), ribonuclease A (32, 55), tropomyosin (30), and insulin (29). Comparison of solution and crystal Raman spectra shows only minor changes for lysozyme (56), ribonuclease-A (57), α-lactalbumin (37), basic pancreatic trypsin inhibitor (59), and carboxypeptidase A (57), but lyophilization of these proteins, and of chymotripsinogen A and ovalbumin (60), results in significant changes both in main-chain and side-chain conformation. Angiotensin II displays the same antiparallel β-conformation in both solid state and concentrated aqueous solution (61). Bovine serum albumin (62), myosin (63), opsin (63a), and an antifreeze glycoprotein of an Antarctic fish (64) have been studied in solution.

Several snake venom proteins have been studied (34, 37, 66, 65). The neurotoxins show β-structure predominantly (34, 66, 65), whereas a cardiotoxin from the Mojave rattlesnake is mainly α-helical (37). A sea anemone toxin, which is both neuro- and cardio-active, has a spectrum dominated by buried tryptophan, and a disordered backbone (66). Protein Raman spectra have been examined in lenses from both avian (38) and bovine (67) eyes. The avian protein is chiefly α-helical, whereas the bovine protein has extensive β-structure. Scanning of individual locations of the bovine lens showed a uniform backbone structure but a definite variation in phenylalanine/tyrosine ratio. Two S–H stretches were detected, at 2582 cm⁻¹ and 2560 cm⁻¹, suggesting different sulfhydryl environments. Prolonged oxidation showed only slight loss in ν_{S-H} intensity, suggesting that the –SH groups are sufficiently restricted in their mobility that disulfide cross-links do not form.

Membrane proteins have been examined in erythrocyte ghosts (68, 69), sarcoplasmic reticulum (70), thymocyte plasma membrane (71, 71a), and transformed lymphocytes (72). Spectra of filamentous bacterial viruses (73) indicate that the coat proteins are α-helical. The peptide ionophores valinomycin (74, 75) and gramicidin (76) have been examined, and changes on complexation observed. A conformation change from β-sheet toward disordered structure is observed when rabbit anti-ovalbumin is precipitated with its antigen (77).

RESONANCE RAMAN PROBES OF CHROMOPHORIC SITES

Normal and resonance Raman spectra are obtained with the same spectrometer. The only requirement for RR spectroscopy is that the laser wavelength fall in or near the absorption band of interest. There are, however, experimental difficulties associated with the measurement of light scattering in an absorption band. These include absorption, heating, photoreactions, and fluorescence. Absorption requires an illumination geometry that minimizes the optical path through the sample, plus optimization of the concentration (78, 79). Laser-induced heating can be minimized by cooling the sample and by rotating (80) or circulating (81) it through the laser beam. Photoreactivity is a more difficult problem, but it has recently been demonstrated (82, 83) that Raman spectra can be obtained, even for highly photoactive species in a high-velocity stream. A sufficiently short transit time through the laser beam keeps the fraction of photolyzed sample to acceptable levels. Fluorescence is the most aggravating interference, since even low levels of photoemission can obscure the inherently weaker Raman scattering. Frequently luminescence arises from impurities, which can be removed by purification procedures or occasionally burned out by laser irradiation. Many, but by no means all, biological chromophores have acceptably low intrinsic fluorescence quantum yields, whereas in other cases addition of external quenching agents (e.g. sodium iodide) sometimes helps. Some improvement may be afforded by time-resolution of the detected light, since scattering is much faster than fluorescence. The needed instrumentation is still under development (84, 85). Perhaps the greatest promise for fluorescent systems comes from a new technique, coherent anti-Stokes Raman scattering (CARS) (86), in which the Raman signal is generated as a coherent beam of light, easily separated from the nondirectional fluorescence. Resonance enhancement can still be exploited, and resonance CARS spectra of dilute aqueous cytochrome c and vitamin B-12 have recently been obtained (87).

The advantage of RR spectroscopy for biological studies is that it offers a sensitive and selective probe of chromophore structure. Only certain

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vibrational modes are enhanced, however. It is important to understand the enhancement pattern in order to make most effective use of the technique and to anticipate its limitation. The theory of RR scattering (88, 89) is complex, and is not reviewed here. It is possible, however, to draw from it a simple and useful generalization (90): significant enhancement can be expected for vibrational modes whose forms (normal coordinates) most closely resemble the distortions experienced by the molecule in the resonant excited state. Thus, $\pi \to \pi^*$ transitions, which produce the dominant absorption bands of polyene and aromatic chromophores, show greatest enhancement for stretching vibrations of the π bonds, which are weakened in the excited state. Likewise, ligand→metal charge-transfer transitions, often exhibited by metalloproteins, provide enhancement of the stretching vibrations of the ligand-metal bonds, which are weakened in the excited state, or of internal ligand vibrations, whose force constants are affected by the loss of the transferred electrons.

The frequencies of the observed RR bands are ground-state vibrational frequencies; only their intensities depend on the properties of the excited state. Biochemical events, e.g. ligand or substrate binding or protein conformation change, alter the vibrational frequencies only to the extent that the ground-state chromophore structure is distorted; such frequency shifts can be interpreted through the method of normal coordinate analysis (14). Intensity changes can occur without any ground-state distortion and are more sensitive monitors of chemical interactions, although structural interpretation is difficult.

$\pi \to \pi^*$ Chromophores

RHODOPSIN The $\pi \to \pi^*$ transitions of conjugated polyenes provide strong enhancement of Raman modes involving the skeletal vibrations of the carbon chain (91, 92). Good quality spectra can be obtained for rhodopsins, using dye laser excitation (93–95). For bacteriorhodopsin, whose photochemistry is reversible, it was possible to show, from the frequency of the C=N Schiff base link of the retinal chromophore, that the nitrogen atom is protonated in the ground state, but deprotonated in one of the photointermediates (94). This feature is plausibly related to the mechanism of the proton pump of the purple bacterial membrane. The irreversible photochemistry of mammalian rhodopsin makes it difficult to ascertain the exact composition of the sample in the laser beam. Oseroff & Callender (96) circumvented this problem by using low temperatures, where the photochemistry is reversible, and two laser beams, one to pump the sample to a particular stationary state composition and the other to generate the Raman spectrum. They were able to show that the Schiff base linkage of rhodopsin is protonated as previously suggested from work at higher temperature (93),

and that the first photointermediate, pre-lumirhodopsin, gives new Raman bands, in the ~ 800 cm^{-1} region, which are not characteristic of all-*trans* retinal. Good quality spectra of ground-state rhodopsin have been generated with rapid flow techniques (82, 83).

HEME PROTEINS   Metalloporphyrins have two low-energy $\pi \rightarrow \pi^*$ transitions. Because they interact strongly, the RR spectra contain a rich assortment of bands of different symmetry types, and quite detailed vibrational assignments can be made (97).

The most intense Raman bands arise from high-frequency (1100–1650 cm^{-1}) porphyrin ring modes. Some of these display characteristic frequency shifts when the heme oxidation or spin state is changed. Back-donation of d electrons from low-spin FeII into the porphyrin π^* (antibonding) orbitals is relieved upon oxidation, or upon attachment of π-acid axial ligands, resulting in increased frequency for the "oxidation state" marker bands. The shifts for O_2 binding to hemoglobin or myoglobin are fully as large as for oxidation to low-spin FeIII derivatives, suggesting that transfer of an electron from FeII to O_2 is essentially complete (97, 98).

A switch from low- to high-spin FeII heme produces larger frequency decrements in a different set of bands, the "spin state" marker bands. These shifts are thought to result from loss in porphyrin π-conjugation accompanying the tilting of the pyrrole rings, as they follow the iron atom out of the plane, a movement that structural studies have firmly associated with the spin-state change (99, 100). This interpretation of the frequency shifts is supported by preliminary results of normal coordinate analysis (101). The observation that these shifts are the same for deoxyhemoglobin (Hb), deoxymyoglobin (Mb), and reduced horseradish peroxidase (HRP) and cytochrome c', as well as for a protein-free five-coordinate FeII heme generated in solution (98), implies that the porphyrin conformation is the same in all of these instances, and that any molecular tension generated by the quaternary structural constraints in deoxyhemoglobin is not expressed in an increased doming of the porphyrin ring, as had been proposed (102).

On the other hand, the spin-state frequency shifts are observed to be significantly greater in the FeIII (met) forms of Hb and Mb than in HRP or cytochrome c' or protein-free FeIII hemes (98). From structural data on high-spin FeIII porphyrins (100), it appears that the doming that occurs in this oxidation state is slight, despite iron atom displacements that are nearly as large as for FeII. The extra shifts observed for Met-Hb and Mb suggest that the globin pocket resists relaxation of the deoxy porphyrin conformation. The implied strain in the resulting FeIII heme provides a structural basis for the inhibition of FeII heme oxidation, which is an essential characteristic of these O_2 binding proteins.

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The heme RR spectra also show weak low-frequency bands, some of which may involve Fe-ligand stretching (102a). These have yet to be analyzed in detail. The Fe–O stretching mode has been identified in oxy-Hb via $^{18}O_2$ substitution (103). Its relatively high frequency, 567 cm^{-1}, suggests an unusually strong bond, consistent with the short Fe–O distance found for the O_2 adduct of Collman's "picket fence" porphyrin (104). The Fe–F stretching mode of FeIII octaethylporphyrin fluoride has been identified at 595 cm^{-1}, using the $^{54}Fe/^{56}Fe$ isotope shift (105). This band does not appear in fluoro-met-Hb, presumabaly because the F$^-$ is weakly bound on the distal side of the out-of-plane heme. Axial ligand modes of bis-pyridine FeII heme are enhanced via resonance with an absorption band that is assignable to FeII $\to$ pyridine charge transfer (98).

Protoheme proteins, such as Hb, Mb, HRP, and *b*-type cytochromes have peripheral vinyl substituents, conjugated to the porphyrin π system, which in *c*-type cytochromes are replaced by saturated thioether links to the protein. Protohemes show extra vinyl-induced RR bands (106), and their visible absorption bands are somewhat red-shifted. By adjusting the laser wavelength it is possible to excite selectively either cytochrome *c* or the *b* cytochromes in succinate-cytochrome *c* reductase (107). Cytochrome *c* oxidase contains heme *a*, which has a peripheral formyl as well as a vinyl group. Preliminary RR spectra show an extra band, at 1660 cm^{-1}, in the reduced form of cytochrome oxidase, which is suggested to be the formyl C=O stretch (108).

CHLOROPHYLLS   Chlorophylls *a* and *b* show many RR features reminiscent of the porphyrins (109, 110). The 9-keto C=O stretch is seen, and it shifts upon aggregate formation, as had been determined from infrared studies (110a). The RR spectra of chloroplasts, dominated by antennae chlorophyll, show several C=O components, suggesting varied environments. Preliminary spectra of bacterial photoreaction centers, as well as of bacteriochlorophyll and its pheophytin, have been reported (111).

VITAMIN B–12   Cobalamins have RR spectra (112, 113) that are dominated by a single intense band near 1500 cm^{-1}, reminiscent of polyenes, although many weaker features are observable. No changes of any significance are observed on changing the sixth ligand, among OH$^-$, CN$^-$, $S_2O_3^{2-}$ cysteinyl, methyl, and 5' deoxyadenosyl (coenzyme B_{12}) (114). Reduction to CoII (B_{12r}) or CoI (B_{12s}) does produce substantial changes in relative intensities.

ULTRAVIOLET CHROMOPHORES   The development of practical ultraviolet lasers holds promise of a major expansion in RR applications. It is

now possible to obtain usable power at 2573 Å by frequency doubling the 5145-Å argon laser line. RR spectra with this wavelength have been reported for adenine monophosphate and uridine monophosphate (115). Similar spectra should be obtainable for tyrosine and trytophan, although fluorescence may present difficulties. Even amide modes are significantly enhanced (24) at 2573 Å, presumably via the $\pi \rightarrow \pi^*$ transition at $\sim$200 nm.

Metal Ion Sites

The characteristic colors of transition metal ions are usually associated with electronic transitions within the manifold of the valence d orbitals, the so-called "d-d" or ligand field transitions. These are orbitally forbidden, and the absorption bands to which they give rise are weak. Resonance Raman enhancement is proportional to the oscillator strength of the electronic transition, and enhancement is expected to be weak for d-d scattering. Indeed centrosymmetric complexes have been shown to exhibit de-enhancement in d-d bands, due to interference effects (116). For noncentrosymmetric complexes, mixing in of p orbitals can significantly increase the oscillator strength. The most interesting biological examples are provided by Co^{2+}, which can substitute for Zn^{2+} (which, having a filled d sub-shell has no d-d transitions of its own) at the low-symmetry active sites of many zinc metalloenzymes, producing d-d bands in the red region of the spectrum with molar absorptivities of several hundred. A preliminary Raman study of Co^{2+} carboxypeptidase and carbonic anhydrase gave negative results (117), but in view of the significant resonance enhancement of Co-ligand stretching modes recently reported (118, 119) for simple tetrahedral Co^{2+} complexes, this application merits further study.

If the transition metal ion is easily reducible, and a bound ligand is easily oxidizable, then intense ligand $\rightarrow$ metal charge-transfer transitions may occur in the visible or near-ultraviolet region. In biological systems these are observed for Fe^{3+} and Cu^{2+} complexes primarily, with ligands such as thiolate, phenolate, and peroxide. Several RR applications are described below.

IRON-SULFUR PROTEINS   The iron-sulfur proteins contain Fe^{3+} and/or Fe^{2+} bound to thiolate (cysteine) and/or sulfide. Those forms containing one or more Fe^{3+} ions have intense absorption in the visible region, attributable to S$\rightarrow$Fe charge-transfer transitions. Resonance enhancement is therefore expected for S–Fe vibrational modes. This was first demonstrated (8, 120) for oxidized rubredoxin, which has one Fe^{3+} ion in somewhat distorted tetrahedral coordination by four cysteine side chains (121). The RR spectrum shows bands at 314 and 368 cm^{-1} assigned to the symmetric and

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nonsymmetric stretching modes of the FeS_4 unit, and bands at 150 and 126 cm^{-1}, possibly arising from bending modes.

Adrenodoxin is a member of the class of proteins that are thought to contain two iron atoms bridged by sulfide ions, and bound to the protein through two cysteine side chains, each iron atom again having a roughly tetrahedral FeS_4 coordination group. The RR spectrum of oxidized adrenodoxin (122) shows three bands in the Fe–S stretching region, at 397, 350, and 297 cm^{-1}. The first and last of these can be assigned to the bridging sulfides by virtue of their large shifts to lower frequency on replacement of sulfide by selenide.

Several proteins contain Fe_4S_4 clusters, in which the iron and sulfur atoms are arranged at the alternating corners of an approximate cube, with each iron atom bound to a cysteine side chain. HiPIP (high-potential iron protein) (123) and bacterial ferredoxin (124) have been characterized by X-ray crystallography. Chemical analogs with a variety of thiolate ligands bound to the Fe_4S_4 cluster have been prepared and characterized (125–127). Much physical evidence suggests that the dianionic form of these analogs is at the same oxidation level (formally $Fe^{2.5+}$) as reduced HiPIP and oxidized ferredoxin. Preliminary RR spectra have been compared for these three species (128). The chemical analog (phenylsulfide derivative) shows two polarized bands, at 332 and 275 cm^{-1}, assignable to symmetric stretching of terminal and bridging Fe–S bonds, respectively. In the protein spectra, the terminal Fe–S mode is split, implying inequivalence of the Fe-cysteine links. This structural feature is of interest in connection with the question of protein control of the redox potential.

TRANSFERRIN   The serum transport protein, transferrin, avidly binds Fe^{3+} with the development of a strong absorption band centered at ~465 nm. Both histidine and tyrosine side chains have been implicated as binding groups, and the visible absorption is attributable to phenolate→ Fe^{3+} charge transfer (129). RR spectra (129–131) show several high-frequency modes, all of which can be assigned to phenolate ring modes, by comparison with the spectrum of a Fe^{3+} complex of an EDTA-like chelate with two phenolate binding groups (129).

HEMERYTHRIN   Hemerythrin, the oxygen-carrying protein of marine worms, binds one O_2 molecule per two iron atoms (132). The deoxy binding site contains Fe^{2+} ions, while the oxy site contains a pair of magnetically interacting Fe^{3+} ions, presumably generated by a reversible reduction of bound O_2 to O_2^{2-}. This view has been confirmed by RR spectroscopy (133) where excitation in the visible absorption band of oxy-hemerythrin reveals

a Raman band at 844 cm^{-1}, shifting to 798 cm^{-1} on substitution of $^{18}O_2$, and therefore assignable to O–O stretching. The frequency is characteristic of peroxides. The Fe–O stretch is also observed at 500 cm^{-1}, shifting to 428 cm^{-1} on $^{18}O_2$ substitution. Moreover, the mode of O_2 binding has been shown to be unsymmetrical (presumably attachment to one or both Fe atoms is through only one of the O atoms) by use of the mixed isotopic molecule $^{16}O^{18}O$ (133a). End-on binding of N_3^- is suggested for metazido-hemerythrin by its RR spectrum (134), which reveals the asymmetric azide stretch to be at 2049 cm^{-1}, close to the frequency 2045 cm^{-1} found in the infrared spectrum of metazidohemoglobin (17).

HEMOCYANIN The oxygen carrier of arthropods and molluscs, hemocyanin, is a copper protein, but its chemistry is analogous to heme-rythrin. Thus, one O_2 molecule is bound per two Cu atoms, with concomi-tant oxidation from Cu^+ to Cu^{2+}. Again RR spectroscopy (135, 136) demonstrates the presence of bound peroxide, with the O–O stretch located at 742 cm^{-1} and shifting to 704 cm^{-1} on $^{18}O_2$ substitution. This mode is in resonance (136) with the visible absorption envelope at $\sim$570 nm, assignable to $O_2^{2-} \rightarrow Cu^{2+}$ charge transfer. Another mode, near 280 cm^{-1} and attribut-able to Cu-imidazole stretching, is observed to be subject to pre-resonance enhancement by an intense near-ultraviolet transition at 340 nm (136). Excitation directly into the 340-nm band, using the 351.1-nm Ar$^+$ laser line (137), produces a RR spectrum with several features below 400 cm^{-1} in the Cu^{2+}-ligand region, but none at higher frequency where imidazole ring modes are expected. This observation militates against assignment of the near-ultraviolet transition to imidazole$\rightarrow Cu^{2+}$ charge transfer (136) and favors assignment to a simultaneous pair excitation (138), which might be expected to enhance only vibrations localized on the Cu^{2+} ions.

"BLUE" COPPER PROTEINS Several copper proteins, e.g. azurin, stel-lacyanin, and plastocyanin, show intense absorption near 600 nm in their oxidized form, giving them an unusually strong blue color. The multi-copper oxidases, laccase and ceruloplasmin, also contain "blue" sites. Much recent evidence (139) points to cysteine$\rightarrow Cu^{2+}$ charge transfer as the origin of the 600-nm absorption band. Infrared data (140) implicate a peptide group in the Cu^{2+} coordination sphere, and NMR evidence points to the involvement of imidazole (141). RR spectra (142–144) show several bands near 400 cm^{-1}. The region near 400 cm^{-1} is appropriate for Cu–N and Cu–O stretching modes. Weak resonance enhancement is found (142) for higher-frequency modes identifiable with peptide group frequencies, consistent with the view that the Cu^{2+} ion is bound to peptide. The $\sim$275 cm^{-1} band

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has been assigned to Cu^{2+}–S stretching, and its weakness has been rationalized with orbital overlap arguments (143, 144). This frequency is also appropriate for Cu^{2+}–imidazole stretching, and it is conceivable that the Cu–S bond is unusually strong, so that its frequency falls in the ~400 cm^{-1} group of bands.

Resonance Raman Labels

Many biological sites are not chromophoric and are not directly amenable to RR investigation. They can, however, be labelled with exogenous chromophores that can probe structural features via their RR spectra. Aromatic azo compounds with appropriate functionality have been used to probe hapten binding to antibodies (145) and inhibitor binding to carbonic anhydrase (146) and to trypsin (147). RR spectra of a chelating azo-dye, zincon, which is a competitive coenzyme inhibitor of liver alcohol dehydrogenase, have been interpreted as indicating binding to the active-site zinc ion through the azo and phenolic groups (148). RR spectra of 4-amino-nitro-3-*trans*cinnamoyl α-chymotrypsin, a stable intermediate, gave evidence of twisting about the C–NO$_2$ bond but no perturbation of the *trans* –C=C– conformation or the coplanar phenyl ring (149).

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